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The underlying reason for failure to cure patients diagnosed with breast cancer is the presence of micrometastases. The stimulation of anti-tumor immune responses represents one of the most effective ways to treat low tumor burdens that are clinically occult. The objective of our proposal is to determine whether a poliovirus replicon vaccine strategy induces systemic immunity and eradication of micrometastases.

During the second year of our project, we have characterized poliovirus replicons which express CEA or HER2/neu. Mice given these replicons generate anti-CEA or HER2/neu antibodies. We have demonstrated protection from tumor challenge in the mice which have antibodies to CEA. The significance of these studies is that we have now characterized the appropriate replicons that will be used to establish route of administration for generating an immune response against CEA or HER2/neu prior to tumor challenge. We are developing companion replicons which express biologically active immune modulators (GMCSF and IL-2) to increase the immunogenicity of the replicons encoding CEA or HER2/neu. The results of these studies will provide essential preclinical observations that will be relevant to the starting of human breast cancer trials targeted against cells that express CEA or HER2/neu.

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Casey D. Moore 9/11/97

PI - Signature Date

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Department of the Army
Annual Report
Grant No. DAMD17-94-J-4403

INTRODUCTION (Rational taken from proposal).

The frequency of micro-metastatic cancer deposits has correlated with a number of factors at the time of primary therapy of breast cancer including regional lymph node metastasis, size of primary tumor, tumor differentiation and certain molecular aspects of individual tumors including aneuploidy, DNA synthesis (S phase) and presence or absence of amplified gene expression for c-myc, p53, or HER2/neu. The use of systemic hormonal therapy, chemotherapy or combined adjuvant therapy directed at these micro-metastases has produced a reduction in metastatic relapse rate and improved survival. These effects are modest, and the majority of patients with micro-metastasis still have disease recurrence and ultimately die of metastatic breast cancer. **Thus, it is clear that additional strategies to eradicate micro-metastasis at the time of initial diagnosis is a major priority in breast cancer therapy research.** It should also be noted that our inability to detect occult micro-metastases means that patients with and without micro-metastases will be treated. The strategy should have low toxicity, ease of administration and low cost.

Multiple animal model studies (6, 7) have demonstrated that immune responses to tumor associated antigens can have dramatic antitumor effects, but such treatment strategies rapidly lose efficacy as progressive tumor growth occurs. Thus, the induction of an immune response to tumor-associated antigens in humans is likely to have limited success in patients with obvious metastases, and its optimal application would be at the time of occult micro-metastasis as an adjunct to primary therapy. Active specific immunotherapy to enhance host immune response to tumor associated antigens has been called "vaccine" therapy, although this application does not fit the strict (narrow) definition that entails prevention of disease rather than therapy of an existing disease. **It is the purpose of this project to define a novel strategy to enhance antitumor response to tumor-associated antigens, leading to the development of therapeutic vaccines.**

A variety of studies have demonstrated that immunization with tumor preparations can produce antitumor immune responses in animal models (4, 5, 12). Similar studies in man have shown antitumor effects in patients with melanoma (3, 10) and other tumors (9). The ability to isolate and clone putative tumor antigens provides the opportunity to utilize more defined reagents and to allow analysis of specific immune responses in guiding the design of active immunotherapy trials. A variety of potential targets include CEA, HER2/neu, MUC-1, MAGE 1, mutated RAS, mutated p53, etc. (7). A number of genetically engineered cancer vaccines utilizing cloned tumor associated antigens in vaccinia virus constructs or with adjuvants are undergoing clinical trials (1). This approach represents a fertile and novel new technology, and that we are just beginning to identify potential tumor associated antigens (and their genes) which will be applicable to novel strategies for enhancement of anti-tumor immune responses in animal models and man.

Poliovirus Replicons to Express Foreign Genes.

The proposed experiments are based on the use of poliovirus as an expression vector for proteins to deliver antigens to immunoreactive sites of the immune system. Poliovirus is attractive for use as a vector for cancer vaccines for several reasons. First, it is an RNA virus with no DNA intermediates in replication. Thus, we can formulate vaccines with oncogenes (e.g. HER2/neu) without concern for cellular transformation (8). Second, a unique vector system based on poliovirus for the expression of foreign genes has been developed by my laboratory. To date, we have constructed poliovirus genomes encoding foreign proteins, referred to as replicons, for over twenty different genes. We have also developed the procedure for complementation of these replicons by providing the capsid protein *in trans*. We are able to generate stocks of encapsidated replicons which encode foreign proteins and which, upon infection of cells, express this recombinant protein. We have demonstrated that administration of replicons alone to experimental animals results in production of an immune response to the foreign protein (11).

BODY OF THE PROPOSAL

The Specific Aims of the proposal have not changed and are as follows:

1 To construct poliovirus replicons which express native and truncated CEA proteins (including secreted and non-secreted molecules (months 1-18).

2. To characterize and optimize the immune response to CEA elicited by both oral and parenteral administration of such vaccines in mice (months 4-24).

3. To test the ability of such poliovirus - replicon CEA vaccines to generate antitumor effects as

measured by resistance to tumor challenge in a syngeneic murine CEA expressing breast cancer model (months 12-36).

4. To test the therapeutic effects of such vaccines in the eradication of breast cancer micro-metastasis in a syngeneic, spontaneously metastasizing CEA positive breast cancer model (months 18-48).

During the first 36 months, we have made consistent progress towards the completion of the designated experiments described in Specific Aims 1-3. To summarize our accomplishments, we have constructed poliovirus replicons which encode several different versions of the CEA protein (2). Three different replicons were constructed and demonstrated to express CEA upon infection of cells. Based on the results of our studies, two replicons were chosen for further analysis. The first replicon contains the complete CEA gene minus the signal sequence (CEA sig-). The second replicon contains the CEA gene lacking the sequences that specify transmembrane anchoring (CEA-TM). Both of these replicons were chosen because of the levels of expression of CEA observed in replicon infected cells. As discussed in our last progress report, we have now extended these studies to another gene relevant to breast cancer, the HER2/neu oncogene. We have constructed a replicon which encodes the extracellular domain of this oncogene. Expression of the HER2/neu protein was confirmed by immunoprecipitation with antibodies specific for the extracellular domain of HER2/neu.

During the last funding period, we have also developed suitable methodologies for large scale production of the replicons encoding the different oncogenes. Included in this work was the development of methodologies to remove both the contaminating vaccinia virus (VVP1) that is used to generate the encapsidated replicons. Safety testing in the transgenic animals demonstrated that no recombinant vaccinia virus was present. In addition, we have also confirmed that no poliovirus exits in these preparations which might have been generated as a results of recombination with VVP1.

Analysis of the immunogenicity of encapsidated replicons encoding CEA or HER2/neu.

Experiments were undertaken during the last two years to analyze the immunogenicity of replicons encoding CEA or HER2/neu. For these studies, we have made use of a mouse which is transgenic for the human receptor for poliovirus (13). This mouse is susceptible to poliovirus when given via intramuscular or intraperitoneal routes. Previous studies from this laboratory have demonstrated that the mouse can be used for analysis of the immunogenicity of encapsidated replicons (11).

Our first series of experiments used groups of 10 mice each. We immunized mice with encapsidated replicons which encode the C-fragment of tetanus toxin as a control. Mice were also immunized with replicons which encode the signal minus version of CEA, which was found to be our optimal expressing replicon from the *in vitro* experiments. We monitored the production of anti-CEA antibodies using a solid phase immunoassay. All mice immunized with the replicon encoding CEA generated an anti-CEA antibody response. The levels of the anti-CEA antibody response were variable, though, with a range of 250 ng of I^{125}-CEA bound per ml of sera to 17,000 ng of I^{125}-CEA bound per ml of sera. Values of approximately 15 ng per ml were found to be background. The variability of the immune response to CEA expressed from a replicon has been noted for other proteins expressed from replicons using these transgenic mice (11).

In a second series of experiments, we analyzed the immunogenicity of encapsidated replicons encoding HER2/neu. Mice were given 10^7 infectious units of encapsidated replicons encoding HER2/neu intramuscularly. Following three immunizations, the sera of the mice were tested for antibodies reacting to HER2/neu. For this analysis, we utilized cell lysates from a cell line known to express high levels of HER2/neu (SKOV.3). The extracts were subjected to SDS-PAGE followed by transfer nitrocellulose membranes. Using the sera from each mouse diluted 1 to 100, we performed a Western blot analysis.

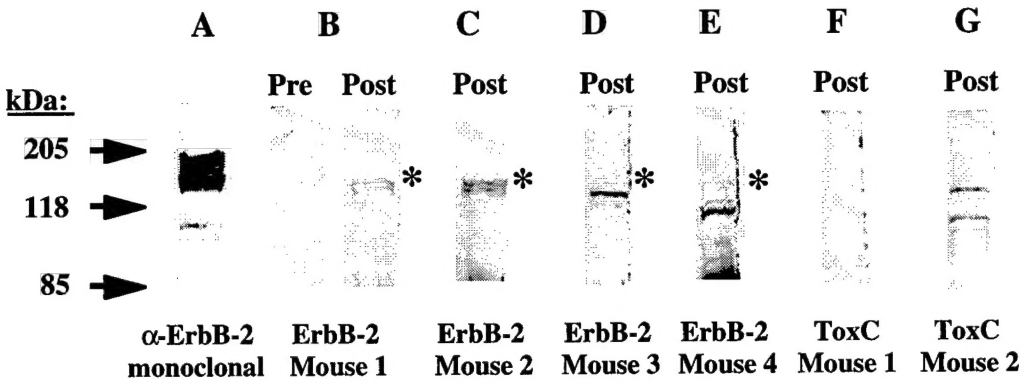

Figure 1. HER2-neu Replicons are Immunogenic in Mice. Mice were immunized a total of three times with replicons encoding HER2-neu or tetanus toxoid C-fragment (ToxC) at three week intervals. After the third immunization, sera were analyzed for HER2-neu-specific antibodies by using a Westen blot assay. A cell lysate from SKOV-3 cells, an ovarian carcinoma cell line that overexpresses human HER2-neu2, was used as the source of antigen on the blot. A monoclonal antibody specific for ErbB-2 was used as a positive control to detect the immobilized HER2-neu from the SKOV-3 cell lysate (Panel A). Sera from mice pre-immunzation did not detect any proteins (Panel B, Pre). Sera collected from four mice post-immunization with ErbB-2 replicons detected a protein consistent with that from the blot probed with the ErbB-2 monoclonal (Panels B-E). This protein was not detected on blots probed with post-immunization sera from mice given ToxC replicons, although other background proteins with a slightly faster mobility were detected at low levels (Panels F and G).

As can be seen, the immunized mice all responded with antibodies which reacted to the HER2/neu blotted onto the nitrocellulose membrane. Similar to what we have found in mice immunized with replicons encoding CEA, a variable level of anti-HER2/neu antibodies were observed among the mice. The results of these studies, though, clearly demonstrate that the replicons encoding HER2/neu are immunogenic when given via intramuscular routes to the transgenic mice.

Tumor Challenge of Immunized Mice.

During the last year's funding, we have demonstrated that inoculation of 2.5 x 10^5 MC38-CEA cells in the flank of the human poliovirus receptor transgenic mice by subcutaneous injection produced a measurable tumor outgrowth in greater than 90% of the mice within 4 weeks. These animals go on to die as a result of tumor burden. To determine if immunization with encapsidated replicons encoding CEA would result in anti-tumor immunity, we immunized 10 mice with 10^7 infectious units of the replicon encoding the signal minus version of CEA (CEA-Sig-). As a control, 10 animals were immunized with the replicon encoding the C-fragment of tetanus toxin. The results of the these challenge studies were very promising. None of the animals immunized with the replicon encoding CEA developed tumors, whereas all of the animals immunized with the control replicon (encoding C-fragment of tetanus toxin) went on to develop tumors and eventually die.

Replicon used for Immunization	Mice with Tumors/ Total Immunized
Not Immunized	8/10
Tetanus Toxoid C-fragment	10/10
Sig$^-$ CEA	0/10

Table 1. Tumor Challenge Studies in Mice Immunized with Replicons. Groups of ten mice were immunized with replicons encoding either the tetanus toxoid C-fragment or Sig-CEA. A third set was not immunized. The groups of mice were given booster immunizations of replicons three times, at three week intervals. After the last booster immunization, the mice were challenged with a tumor cell line (MC38-CEA) that expresses human CEA. The mice then were monitored for tumor development. The mice that developed tumors ultimately died; all of the mice immunized with the Sig-CEA replicon survived the challenge study.

Based on the results of these studies, we performed additional challenge experiments using mice that had been immunized with replicons encoding CEA by intramuscular or intraperitoneal routes. In these experiments, we again monitored the effectiveness of the immunization by measuring the antibodies to CEA in the sera. However, in this case, we noted that the animals did not response to CEA by generation of anti-CEA antibodies. This was perplexing given the positive results obtained during the first immunization trial. Challenge of these animals resulted in tumors in all of the immunized animals. Further studies revealed that our mice transgenic for the human receptor for poliovirus had lost expression of the transgene. The reason for this was unclear. The fact that the animals were not susceptible to infection by poliovirus replicons though explains our negative results. We have recently obtained new transgenic mice from Lederle-Praxis Biologicals. Currently, we are growing additional replicons to re-do the immunization experiments.

Enhancement of the immunogenicity of encapsidated replicons encoding CEA.

In parallel experiments, we have begun to develop replicons which might be of use to enhance the immunogenicity of those replicons encoding CEA. Our strategy here has been to develop replicons which encode biologically active immune modulators (cytokines) that would have utility as a biological adjuvant. Our initial studies have focused on two of the most commonly use cytokines for this purpose: GM-CSF and IL-2. We have constructed replicons which encode the murine genes for GM-CSF and IL-2. Infection of cells with these replicons results in the production GM-CSF or IL-2 as measured by a commercially available ELISA (Quantikine Kit).

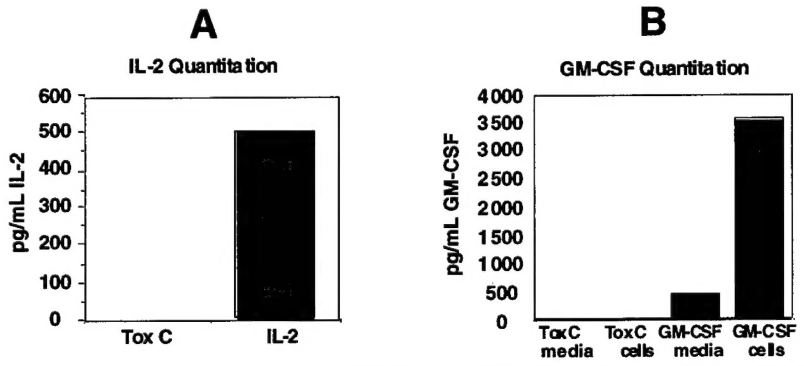

Figure 2. Expression of IL-2 and GM-CSF from replicons. $5x10^5$ Hela H1 cells were infected with replicons encoding either murine IL-2 or murine GM-CSF at a multiplicity of infection of 10 infectious units per cell. Cell lysates and/or media were collected at 6 hours post-infection and analyzed for murine IL-2 (Panel A) or murine GM-CSF (Panel B) by using a commercially available ELISA kit. The graphs show concentrations (pg/mL) of the cytokines detected in cell lysates (IL-2) or both cell lysates and culture supernatants (GM-CSF).

We determined that infection of cells with replicons encoding IL-2 or GM-CSF resulted in the production of 6,000 pg of IL-2 per 10^6 cells or 4,000 pg of GM-CSF per 10^6 cells, respectively. We have also determined that the cytokines produced are biologically active.

In a preliminary experiment, we immunized transgenic mice with soluble tetanus toxoid and the replicon encoding GM-CSF (10^8 infectious units per dose); as a control, mice were given only tetanus toxoid. A total of 2 immunizations were given (i.e. prime and boost). Sera from the mice were collected and the end point titer of antibodies to tetanus toxoid was determined. **Interestingly, we found that, on average, the mice co-immunized with replicons encoding GM-CSF had 3-4 fold higher titers two weeks after the last immunization. Follow-up studies of these mice revealed that the increase in anti-tetanus toxoid titer was maintained for approximately six weeks after the booster immunization.** The results of these preliminary experiments point to the potential for replicons encoding cytokines to enhance the immune response to the target antigen when co-administered with other replicons or antigen.

Experiments are planned to utilize the replicons encoding GM-CSF or IL-2 in conjunction with replicons encoding CEA or HER2/neu to determine if we can enhance the immune responses to these antigens. In addition, we have recently obtained recombinant soluble CEA and HER2/neu protein that will be incorporated into the immunization strategy similar to what we have done in preliminary experiments with tetanus toxoid. We will monitor the immune responses both for antibodies to CEA or HER2/neu as well as T-cell mediated immune response (T-cell proliferation to recombinant CEA or HER2/neu). We will determine if we obtain more consistent

results with our immunization prior to challenge of the mice with the MC38-CEA cells. As a corollary to these experiments, we will also evaluate those animals immunized with HER2/neu for enhanced immune response to this oncogene. We are in the process of developing a tumor challenge system in which the MC38 cells have been transformed with HER2/neu.

Conclusion.

The proposed studies are progressing on schedule. As planned, we have now constructed and characterized the appropriate replicons for expression of CEA. Experiments have been performed to demonstrate the immunogenicity of these replicons. We have found that mice which demonstrate a immune response to CEA are capable of resisting tumor challenge with the MC38-CEA tumor. We were concerned, though, about the variability of the immune response. To address this issue, in parallel experiments, we have developed replicons which encode biologically active immune modulators which can be used to enhanced the immunogenicity of our encapsidated replicons. Experiments are planned during the next year to test the immunogenicity of these preparations within the transgenic mice. The mice will then be challenged with the MC38-CEA expressing tumor. Ultimately, the evaluation of different encapsidated replicons encoding biological response modifiers, as well as CEA, will allow the formulation of a cocktail vaccine approach that can be further evaluated in primates. In parallel experiments, we have found that primates immunized with replicons encoding the C-fragment of tetanus toxin generated a strong anti-tetanus antibody response. Thus, primates might be a better model for immunization with replicons. Towards this goal, we intend to immunize a limited number of primates with replicons encoding CEA to determine if we can generate an anti-CEA immune response.

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